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Overexpression of a novel cold-responsive transcript factor *LcFIN1* from sheepgrass enhances tolerance to low temperature stress in transgenic plants

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Summary

As a perennial forage crop broadly distributed in eastern Eurasia, sheepgrass (*Leymus chinensis* (Trin.) Tzvel) is highly tolerant to low-temperature stress. Previous report indicates that sheepgrass is able to endure as low as −47.5 °C,allowing it to survive through the cold winter season. However, due to the lack of sufficient studies, the underlying mechanism towards the extraordinary low-temperature tolerance is unclear. Although the transcription profiling has provided insight into the transcriptome response to cold stress, more detailed studies are required to dissect the molecular mechanism regarding the excellent abiotic stress tolerance. In this work, we report a novel transcript factor *LcFIN1* (*L. chinensis* freezing-induced 1) from sheepgrass. *LcFIN1* showed no homology with other known genes and was rapidly and highly induced by cold stress, suggesting that *LcFIN1* participates in the early response to cold stress. Consistently, ectopic expression of *LcFIN1* significantly increased cold stress tolerance in the transgenic plants, as indicated by the higher survival rate, fresh weight and other stress-related indexes after a freezing treatment. Transcriptome analysis showed that numerous stress-related genes were differentially expressed in *LcFIN1*-overexpressing plants, suggesting that *LcFIN1* may enhance plant abiotic stress tolerance by transcriptional regulation. Electrophoretic mobility shift assays and CHIP-qPCR showed that LcCBF1 can bind to the CRT/DRE *cis*-element located in the promoter region of *LcFIN1*, suggesting that *LcFIN1* is directly regulated by LcCBF1. Taken together, our results suggest that LcFIN1 positively regulates plant adaptation response to cold stress and is a promising candidate gene to improve crop cold tolerance.

Introduction

As a major environmental stress, low temperature disrupts cellular homeostasis and severely limits both plant productivity and distribution (Gilmour *et al.*, 1988). To cope with low-temperature stress and adapt to seasonal temperature variations, plants have evolved to modulate the expression of specific stress-responsive genes after perceiving stress signals (Chinnusamy *et al.*, 2007; Fujita *et al.*, 2006; Huang *et al.*, 2012; Yamaguchi-Shinozaki and Shinozaki, 2006). Along with the progress of molecular biology and biotechnology, a number of stress-responsive genes have been identified and used to improve plant tolerance to low temperature and other abiotic stresses in important agricultural and cash crops. For example, transgenic rice overexpressing *OsbZIP23*, *SNAC2* and *HvCBF4* confers salinity, low temperature and drought stresses without stunning growth (Hu *et al.*, 2008; Oh *et al.*, 2007; Xiang *et al.*, 2008); transgenic barley overexpressing *TaCBF14*, *TaCBF15* and *OsMYB4* improves frost tolerance and germination rate under low temperature (Soltész *et al.*, 2012, 2013); transgenic wheat overexpressing *GmbZIP1* enhances drought tolerance (Gao *et al.*, 2011), and the aluminium tolerance of barley was enhanced by expressing citrate transporter genes such as *TaALMT1*, *SbMATE* and *Arabidopsis FRD3* (Zhou *et al.*, 2014). In addition, expression of barley *HVA1*, which encodes a group 3 LEA protein, improves drought tolerance in creeping bentgrass (*Agrostis stolonifera* var. *palustris*) and enhances tolerance against cold, drought and salinity in mulberry (*Morus indica* L.) (Checker *et al.*, 2012; Fu *et al.*, 2007).

As a dominant species and perennial forage of Pooideae subfamily, sheepgrass distributes in wide communities on the steppes of eastern Eurasia (Shi and Guo, 2006). Because of its high protein content, palatability and digestibility, perennial sheepgrass is suitable to be used as forage crop species and has been successfully cultivated in Ningxia province in China (Chen *et al.*, 1988; Ma *et al.*, 2013). Moreover, sheepgrass comprises a strong ability to cope with many abiotic stresses, including drought (<6% soil moisture), high salt (600 mM NaCl) and extremely low temperature (−47.5 °C) (Chen *et al.*, 2013).

To understand the mechanism of how sheepgrass resists abiotic stresses, a number of stress-responsive genes from

sheepgrass have been isolated and characterized. Among them, *LcDREB3a* and *LcMYB1* improved salt tolerance in transgenic plants; overexpressing *LcWRKY5* increased the rates of cotyledon greening and plant survival in *Arabidopsis* under drought stress (Cheng *et al.*, 2013; Ma *et al.*, 2014; Peng *et al.*, 2011). Using next-generation sequencing, more than 1500 genes related to high salt stress were identified (Li *et al.*, 2013a,b). Two of them *LcSAIN1* and *LcSAIN2*, which were induced by salt and other various abiotic stresses, have been characterized (Li *et al.*, 2013a, b). Overexpressing *LcSAIN1* in rice improves the greening rate of cotyledons, the fresh weight, root elongation, plant height and survival rate in a salinity condition (Li *et al.*, 2013b). To further understand the stress-response mechanisms, the sheepgrass transcriptome under below-zero temperature was also analysed by next-generation RNA sequencing (Chen *et al.*, 2013). As a result, 2979 unigenes with freezing-specific response were identified (Chen *et al.*, 2013).

In this study, we focused on one of the freezing-responsive unigenes, *LcFIN1*. Expression analysis indicated that *LcFIN1* was rapidly induced by cold stress, suggesting that it may involve in the early response to cold stress. Overexpression of *LcFIN1* in *Arabidopsis* significantly increased tolerance to low-temperatures stress. Transcriptome analysis showed that the expression of some canonical cold-response and highly hydrophilic cold-stress-related genes was increased to a much higher level in *LcFIN1*-overexpressing plants than in the wild-type plants after cold treatment. Our work of *LcFIN1* function initializes the understanding towards the mechanism of sheepgrass adaptations to extreme cold stress and provides a valuable resource to improve forage crops cold tolerance.

Materials and methods

Plant materials and treatments

The Zhongke 1 variety of sheepgrass was grown in a soil mixture containing 2 : 1 peat moss and vermiculite (v/v) in a glasshouse at 22–27 °C with a 16-h light/8-h dark photoperiod (light intensity: 150 μmol/m^2/s). Four-week-old seedlings were used for abiotic stress or abscisic acid (ABA) treatment. For cold treatment, 4-week-old seedlings were exposed to 4 °C. For ABA, dehydration and salt treatments, 4-week-old seedlings were submerged in half-strength Murashige and Skoog (1/2 MS) liquid media supplemented with 100 μM ABA, 20%PEG and 200 mM NaCl, respectively. Seedlings were sampled after 0, 1, 2, 4, 8, 12, 24 and 48 h of treatment. For tissue-specific gene expression analysis, panicle, leaf, sheath, rhizome, root and tiller bud samples were collected from 2-year-old sheepgrass grown in a glasshouse as described above. All tissue samples were immediately frozen in liquid nitrogen and stored at −80 °C.

Tobacco (*Nicotiana benthamiana*) seeds were grown in pots with 2 : 1 peat moss and vermiculite soil (v/v) in a growth chamber as described above. *Arabidopsis thaliana* (Columbia) seeds were surface-sterilized by incubation in 75% ethanol for 3 min, 20% NaClO for 6 min, followed by 3–5 washes with sterile water. The sterilized seeds were germinated on MS media (pH 5.8) supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar and then grown in chambers as described above after 2 days in darkness.

RNA isolation and real-time RT-PCR

Total RNA was isolated using the Trizol Kit (TaKaRa, Dalian, China) according to the manufacturer's instruction. PrimeScript™ RT reagent Kit (TaKaRa, Dalian, China) was used for first-strand cDNA synthesis and further quantitative real-time PCR reaction following the manufacturer's instruction. Real-time PCR was carried out on LightCycler 480 real-time PCR system (Roche, Rotkreuz, Switzerland) using the programme of 40 cycles (95 °C for 5 s and 60 °C for 20 s). The $2^{-\Delta\Delta CT}$ method was used to analyse the data (Livak and Schmittgen, 2001). Quantitative real-time PCR primers are listed in Table S1.

LcFIN1 cloning and bioinformatics analysis

To obtain full-length LcFIN1 cDNA, RNA from 4-week-old sheepgrass seedlings was used for RACE. First-strand cDNA was synthesized using the SMART™ RACE cDNA Amplification Kit (Clontech, Palo Alto, CA) according to the manufacturer's instructions. *LcFIN1* F1 and R1 RACE primers were used to amplify 5′ and 3′ ends. Putative full-length *LcFIN1* cDNA and genomic DNA were amplified using gene-specific primers F2 and R2. *LcFIN1* cDNA was cloned into the pEASY-T5 Zero vector TransGen (Beijing, China) for sequencing by Biomed (Beijing, China).

LcFIN1 homologues were identified by sequence similarity searching in NCBI database using BLAST program. Multiple sequence alignment and amino acid sequence phylogenetic analysis were performed using the DNAMAN v5.0 program with observed divergency method (Lynnon Biosoft Inc., Vandreuil, Quebec, Canada). The signal peptide was predicted using SignalP 4.0 program (Petersen *et al.*, 2011), and transmembrane segments were predicted using the TMHMM 2.0 program (Krogh *et al.*, 2001). Conserved protein domains were predicted using Pfam (Finn *et al.*, 2014) and SMART programs (Letunic *et al.*, 2012). LcFIN1 subcellular localization was predicted using the Plant-mPLoc program (Chou and Shen, 2010).

Plasmid construction

To generate the subcellular localization construct, *LcFIN1* coding region was cloned into the pMDC45 vector to express LcFIN1-GFP fusion protein under the control of the cauliflower mosaic virus 35S (CaMV 35S) promoter. To generate the overexpression construct, the *LcFIN1* ORF was cloned into the pK7G2D vector under the control of the CaMV 35S promoter. To express LcCBF1 protein *Escherichia coli*, *LcCBF1* open reading frame was cloned into pET-28a (+) vector to generate the *LcCBF1-His* fusion construct. To construct the effector plasmid used in the transactivation assay, the *LcFIN1* ORF was cloned in between *BamH-I* and *EcoR-I* on the pRT-BD vector. Fidelity of the constructs was confirmed by sequencing. The 5XGAL4-LUC reporter, pPTRL (*Renilla reniformis* luciferase driven by 35S promoter) internal control, pRT-BD expression vector and the 35S-BD-VP16 positive-control plasmids were kindly provided by Dr. Jinsong Zhang.

Plant transformation

Transgenic construct was introduced into *Agrobacterium* strain EHA105 for plant transformation. Floral dip method was used for *Arabidopsis* transformation as previously reported (Clough and Bent, 1998). Transgenic plants were selected on MS media supplemented with 50 mg/L hygromycin (Roche). T1 transgenic plants that segregated in a ratio of approximately 3 : 1 for hygromycin resistance were presumed to contain a single insertion. The positive lines were further confirmed by PCR using F3 and R3 primers. *LcFIN1* transcript levels in T3 transgenic plants were determined by quantitative real-time PCR, and *AtActin* (AY096397) was used as normalizer. Three transgenic lines exhibiting relatively high expression were selected for further study.

LcFIN1 subcellular localization

To study the subcellular localization of LcFIN1, *pMDC45 -LcFIN1-GFP* construct was introduced into *Agrobacterium tumefaciens* strain EHA105. The tobacco leaf was infiltrated by the *Agrobacterium* to express the GFP fusion protein. Following incubation in growth chambers for 2–3 days, GFP fluorescence in tobacco leaves was imaged by confocal microscopy (Leica, Göttingen, Germany) using a 60× water-immersion objective. The p19-GFP (Voinnet *et al.*, 2003) was used as the positive control.

LcFIN1 transcriptional activity assay

For transient expression assays, the reporter, effector and internal control plasmids were cotransformed into *Arabidopsis* protoplasts. Following incubation in weak light for 16 h, LUC activity was detected using the Dual-luciferase reporter assay system (Promega, Madison, WI). Relative reporter gene expression levels are determined by ratio of LUC activity to pPTRL levels (Wei *et al.*, 2009).

Chilling- and freezing-tolerance assays

To investigate the chilling tolerance of *LcFIN1* overexpression and wild-type *Arabidopsis*, seeds from transgenic and wild-type plant were surface-sterilized as described above and then sown side-by-side on MS media. The agar plates were placed at 4 °C for the desired time periods. For the germination assay, the percentage of germinated seeds was calculated using the number of visible radicles appearing obvious difference between wild-type and transgenic plants at 4–5 weeks. For the hypocotyl elongation assay and the measurement of radicle (primary root) length, seeds of transgenic and wild-type plants were first germinated at 22 °C for 4 days and then transferred to 4 °C for 10 weeks, and the hypocotyl elongation and root length were used as metric to evaluate chilling tolerance. Seeds grown at 22 °C were used as a control.

To evaluate freezing tolerance, 3-week-old plants grown under normal growth conditions were cold-acclimated at 4 °C for 3 days and then transferred to a low-temperature chamber of −8 °C overnight. Plants that were not cold-acclimated were used as the control. For the 6-week-old flowering stage plants, cold stress was performed at −4 °C for 6 h, with or without 24 h of cold acclimation at 4 °C before stress. The survival rate was calculated following 14-day recovery under normal conditions.

Physiological measurements

Two-week-old seedlings grown on MS media were subjected to 4 °C for 12 h. Samples were collected to quantify the soluble sugar and MDA contents as previously described (Kramer *et al.*, 1991; Li *et al.*, 2013a). To determine chlorophyll content, leaves were collected and immerged in 95% ethanol at room temperature overnight in darkness. Chlorophyll concentrations were determined as previously described (Amid *et al.*, 2012). Total superoxide dismutase (SOD) activity was measured using nitro-blue tetrazolium (NBT) reduction as previously described (Durak *et al.*, 1993). Water loss rates were measured as previously described (Du *et al.*, 2012). To assay electrolyte leakage, all rosette leaves of ten 14-day-old seedlings treated with 4, 0, −2, −4, −6, −8 and −10 °C for 1 h were removed and initial conductivities were recorded after immersion in ddH_2O for 2 h with shaking gently. Samples were then boiled for 30 min, and final conductivities were measured after cooling to room temperature (Gilmour *et al.*, 1988).

Electrophoretic mobility shift assay

The electrophoretic mobility shift assay (EMSA) was performed as previously described (Guo *et al.*, 2013). Biotin-labelled DNA (Table S1) was used as the probe, and unlabelled DNA containing the same sequence was used as the competitor.

Transcriptome analysis

Total RNA was isolated from wild-type and transgenic plants using Trizol Reagent according to the manufacturer's instructions (TaKaRa, Dalian, China). The concentration and quality was determined using a NanoDrop 2000 (Thermo Fisher, Waltham, MA). mRNA was purified from total RNA using the Dynabead mRNA purification kit according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). mRNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). Double-stranded cDNA was synthesized using the SuperScript Double-Stranded cDNA Synthesis kit (Invitrogen). Specific adapters were ligated to fragmented cDNA, which was denatured to generate single-stranded cDNA, and used for emulsion PCR amplification for sequencing.

All assembled unigenes were annotated using the TAIR website (ftp://ftp.arabidopsis.org/home/tair/Microarrays/Datasets/). For differential gene expression analysis, the number of reads for each sample was converted to reads per kilobase per million mapped reads (RPKM) (Mortazavi *et al.*, 2008). Following conversion, the DEGseq package MA-plot-based method with random sampling model (MARS) was used to calculate the abundance of each contig in the analysed samples. The FDR (false discovery rate) was used to determine the *P*-value threshold, and a FDR < 0.001 was considered to indicate differential expression abundance. Hierarchical clustering was performed using the CLUSTER program (Eisen *et al.*, 1998) on $\log_2$-transformed data, and the resultant clusters were visualized using the TREEVIEW program (http://rana.lbl.gov/EisenSoftware.htm).

Gene ontology term enrichment was analysed by the GoPipe program using a BLASTP search against the Swiss-Prot and TrEMBL databases (Chen *et al.*, 2005). KEGG pathway annotations were performed using the KEGG Automatic Annotation Server (KAAS) using the bidirectional best-hit information method (Moriya *et al.*, 2007).

Statistical analysis

Analysis of variance (ANOVA) was applied to compare different samples, and Duncan's multiple range test was used to analyse the differences between samples. Statistical analysis was carried out using the SPSS 21.0 program (IBM, Chicago) and a *P*-value of <0.001, <0.01 or <0.05 was considered to be statistically significant.

ChIP assays

LcCBF-or LcFIN1-specific antibody was prepared in rabbits (Genomics). Chromatin preparation and immunoprecipitation (ChIP) assay was performed using the EpiQuik Plant ChIP kit (Epigentek, Brooklyn, NY) with three biological replicates. The enrichment of the DNA fragments was detected by quantitative PCR using SYBR PrimeScript PCR Kit (TaKaRa, Dalian, China) on a real-time system (Roche lightcycler 480). *LcACTIN* from sheepgrass or *AtACTIN2* from Arabidopsis was used as internal control. The data were calculated using the comparative Ct method (Livak and Schmittgen, 2001). The primers are listed in Table S1.

Results

Cloning and sequence analysis of LcFIN1

Partial *LcFIN1* cDNA was identified from previous cold-stressed sheepgrass transcriptional profiling data (Chen *et al.*, 2013). The full-length cDNA was obtained using the RACE technique. LcFIN1 is encoded by a single exon (967 bp) with a 597-bp open reading frame (ORF) (GenBank accession no. KM387398). The deduced LcFIN1 protein comprises 198 amino acids with molecular mass of 21.2 kDa and pI of 5.13. Bioinformatic analysis predicted a DUF761 domain with unknown function at the C-terminus.

Multiple sequence alignment revealed that LcFIN1 is homologous to monocot homologues from *Aegilops tauschii* (87%), *Triticum urartu* (81%) and *Hordeum vulgare* (72%). By contrast, the similarity with homologues from dicots is low (Figure 1a). Phylogenetic analysis also indicated that LcFIN1 is closely related to the homologue from *A. tauschii* and *T. urartu* (Figure 1b). FIN1 homologues in different species are all annotated as predicted or hypothetical proteins, suggesting their unknown function.

LcFIN1 transcript is rapidly induced by cold treatment

To examine the *LcFIN1* response to cold stress, 4-week-old sheepgrass plants were subjected to 4 °C for different intervals. Expression analysis showed that *LcFIN1* transcript level was rapidly increased 13.5-fold after 1-h cold treatment. After 4-h treatment, the transcript level was dramatically increased up to 137.9-folds. After that, the expression level gradually declined (Figure 2a). This rapid and strong induction expression suggested that *LcFIN1* participates in the early response to cold stress. Unlike cold stress, dehydration, salt and ABA were not able to induce LcFIN1 transcription (Figure 2b), suggesting that FIN1 specially responds to cold stress.

Tissue-specific expression analysis indicated that *LcFIN1* widely expressed in all tissues, such as panicle, leaf, sheath, rhizome, root and tiller bud samples. The highest expression was detected in leaf samples, whereas the lowest expression was detected in panicle and tiller bud samples (Figure 2c). As expected, *LcFIN1* transcript levels were significantly increased in each tissue under cold stress.

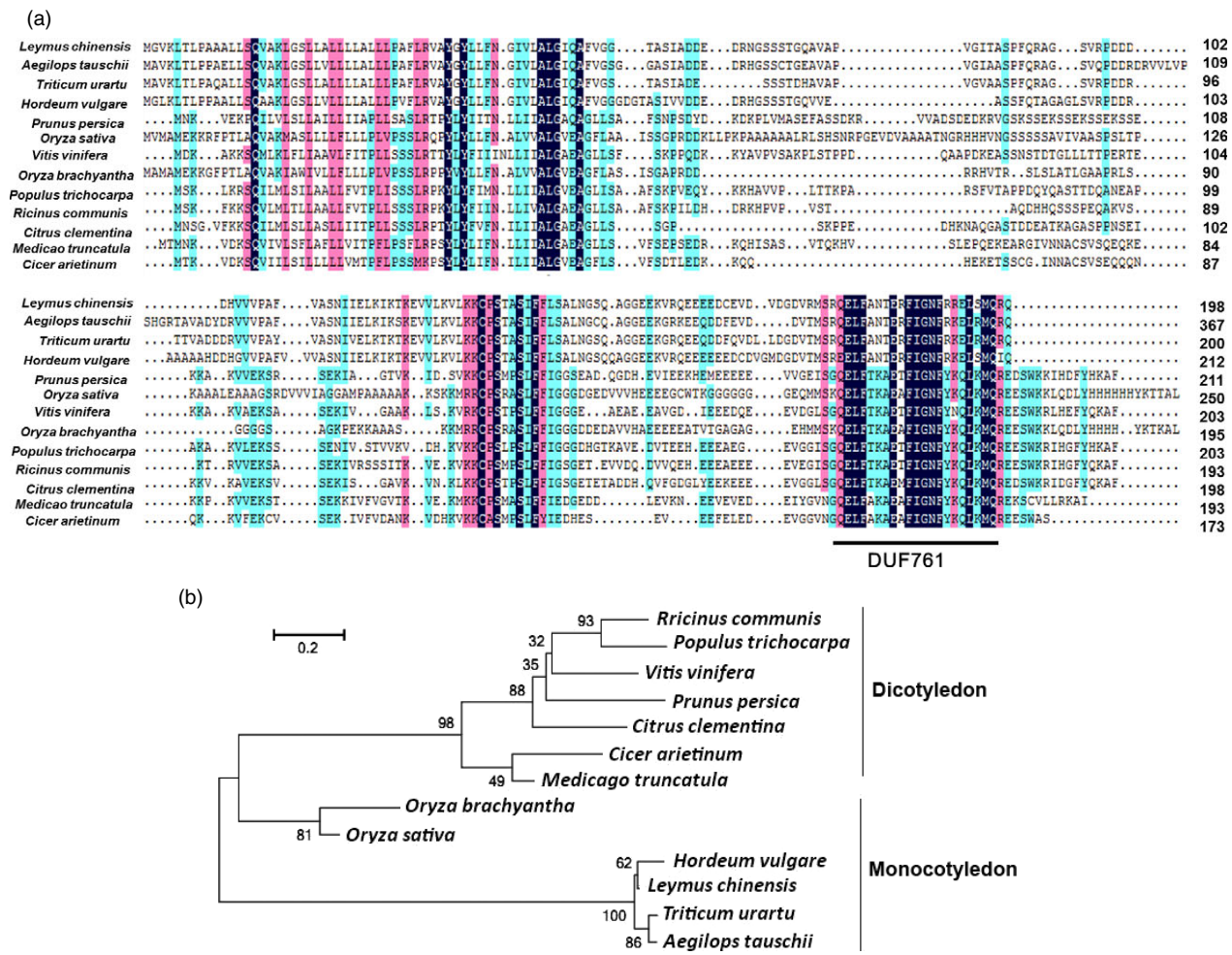


Figure 1 Sequence alignment and phylogeny of LcFIN1 homologues. (a) Amino acid alignment of LcFIN1 homologues. (b) A phylogeny tree was reconstructed using the DNAMAN tree program with observed divergence method based on sequences in (a). Dicotyledon and monocotyledon plant genomes encoding putative FIN1 proteins were grouped.

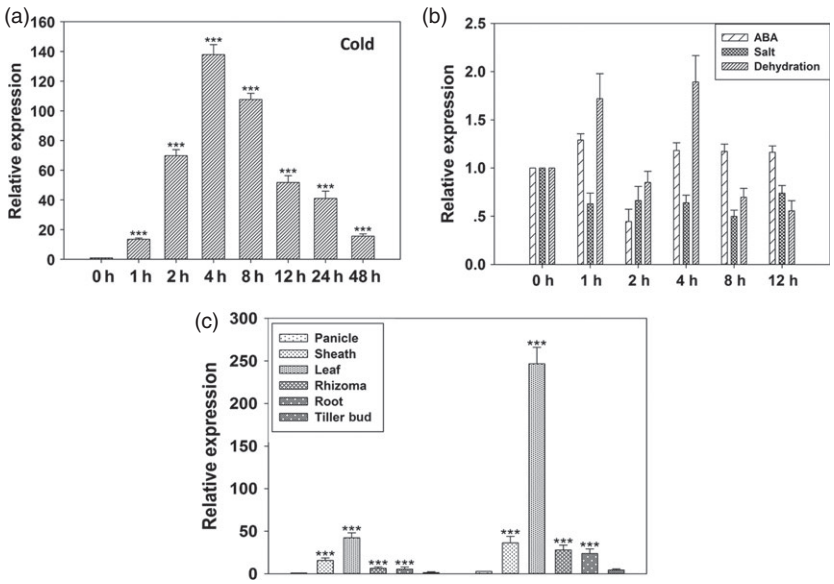


Figure 2 *FIN1* expression patterns. (a) Time course expression of *FIN1* under cold stresses. (b) Expression of the *LcFIN1* was not induced by abscisic acid (100 μM ABA), salt (200 mM NaCl) or dehydration. (c) Tissue-specific expression of *LcFIN1* in response to 12-h cold treatment (4 °C). Transcript levels were determined by quantitative RT-PCR. Error bars indicate standard deviations of three technical replicates. Asterisks represent statistically significant differences between panicle and other tissues (sheath, leaf, rhizome, root, tiller bud) by *t*-test (***$P < 0.001$).

LcFIN1 was localized to the nucleus

To understand LcFIN1 function, we investigated its subcellular localization. As shown in Figure 3, LcFIN1-GFP fusion protein was clearly localized to the nucleus, suggesting that LcFIN1 is a nucleus-localized protein and therefore may participate in downstream gene transcript regulation in response to cold stress.

LcFIN1 has transcriptional activation activity

Given its nucleus localization, we speculated that LcFIN1 may be a novel transcription factor. To confirm this hypothesis, we examined the potential transcriptional activation activity using the *Arabidopsis* protoplast system. As shown in Figure 4, LcFIN1 was able to promote the relative LUC activity, suggesting that LcFIN1 can activate the transcription of the reporter gene. When LcFIN1 was co-incubated with the positive-control VP16 transcription factor in the assay system, LcFIN1 also further increases the VP16-promoted gene expression. Taken together, these results suggested that LcFIN1 has an intrinsic transcriptional activation capacity.

Overexpression of *LcFIN1* in *Arabidopsis* increases tolerance to cold stresses

To further understand *LcFIN1* function, LcFIN1 was overexpressed in *Arabidopsis* under the control of the 35S promoter. More than 20 independent transgenic lines (T1) were obtained. Among these lines, six segregated in a nearly 3 : 1 ratio for the antibiotic selection and were grown for two generations to generate homozygous lines. RT-PCR (Figure 5a) and quantitative RT-PCR (Figure 5b) indicated that *LcFIN1* expression was significantly up-regulated in the six T3 homozygous transgenic lines. Three transgenic lines (L7, L12 and L16) were selected for further study due to their high LcFIN1 expression levels. Under normal growth condition, *LcFIN1* overexpression lines showed no growth difference with wild type, suggesting that overexpression of *LcFIN1* does not cause any aberrant phenotype (Figure 5c,d).

To test the chilling tolerance, we examined seed germination rate for both overexpression lines and wild type at normal condition and 4 °C. Under normal condition, both transgenic lines and wild type germinated well and showed no difference. By

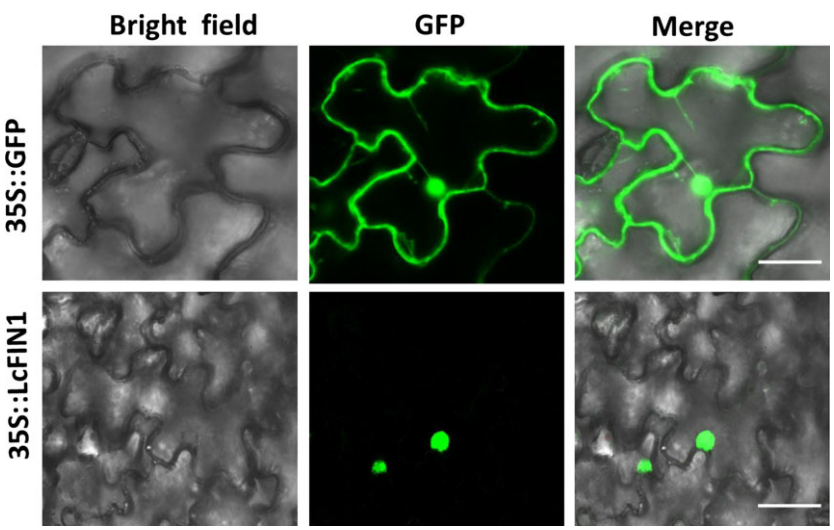


Figure 3 Subcellular localization of LcFIN1 in tobacco epidermal cells. *LcFIN1-GFP* and free *GFP* were transiently expressed in tobacco epidermal cells. Green fluorescence was imaged using confocal microscopy 48 h after *Agrobacterium*-mediated infiltration. Upper row, 35S::GFP; lower row, 35S::LcFIN1-GFP. Left, bright-field images; centre, fluorescence signals; right, overlay. Scale bar: 20 μm.

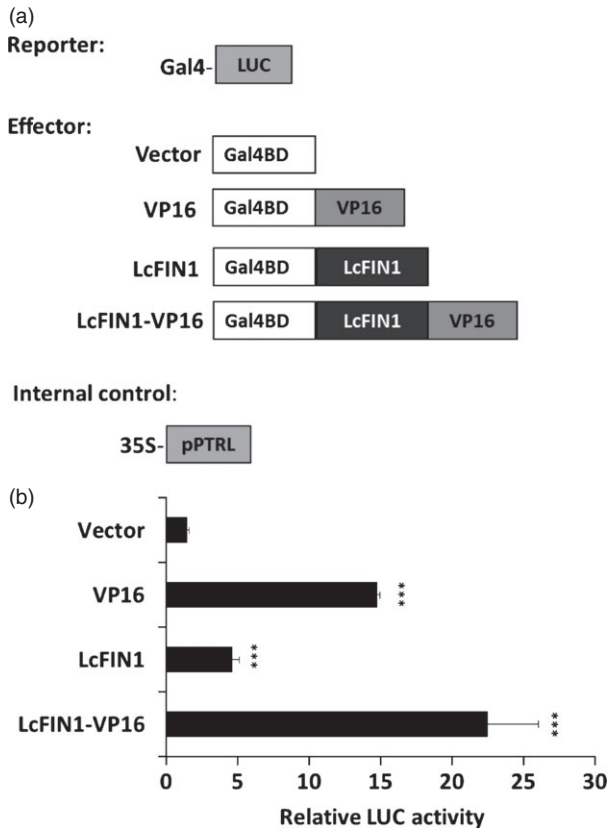


Figure 4 LcFIN1 has transcriptional activation activity. (a) Diagram of constructs used. (b) Effects of LcFIN1 expression on reporter gene activity and VP16-mediated LUC gene expression as revealed by relative LUC activity. Relative reporter gene expression levels are determined by the ratio of LUC activity to pPTRL levels. GAL4 BD (Vector) and VP16 were used as negative and positive control, respectively. Error bars indicate standard deviations of three independent biological replicates. Asterisks represent statistically significant differences between vector and VP16, LcFIN1 and LcFIN1-VP16 by *t*-test (***$P < 0.001$).

contrast, overexpression lines showed much higher germination rate than wild type at 4 °C (Figure 6), indicating that overexpression of *LcFIN1* increases resistance to chilling stress (Figure 6).

In the freezing-tolerance assays for vegetative stage plants at −8 °C, the *LcFIN1* overexpression lines showed less loss of survival rates and fresh weight than wild type regardless of cold acclimation (Figure 7a–c). Additionally, the flower stalks of cold-acclimated *LcFIN1*-overexpressing plants grew faster than those of wild-type plants during the recovery period (Figure 7d). For the plants without cold acclimation, there was no visible flower stalk growth. The fresh weights of transgenic and wild-type plants at the flowering stage showed no significant difference under normal conditions (Figure 8a,b). In contrast, the survival rates of *LcFIN1*-overexpressing plants subjected to −4 °C treatments were significantly higher than those in wild-type plants (Figure 8a,c). Taken together, these results indicated that overexpression of *LcFIN1* promotes freezing tolerance in plants.

Overexpression of *LcFIN1* promotes cold-stress-response physiological traits

Enhanced cold tolerance in *LcFIN1*-overexpressing plants compared with wild-type plants prompted us to measure several important physiological indices such as chlorophyll content which have been widely used to evaluate the plant stress response (Kim *et al.*, 2001). As shown in Figure 9a, the chlorophyll content is relatively higher in the transgenic plants than in wild type under cold or nonstressed conditions, suggesting an increased photosynthetic potential for transgenic plants. We also measured the soluble sugar accumulation, as soluble sugar accumulation can improve abiotic stress tolerance (Konstantinova *et al.*, 2002; Parvanova *et al.*, 2004). In comparison with wild-type plants, soluble sugar contents in the transgenic lines were significantly higher ($P < 0.01$) in both normal and cold conditions (Figure 9b). The enhanced accumulation of compatible solutes may result in lower plant cell water potential, leading to decreased water loss. Consistent with this notion, the three *LcFIN1*-overexpressing plants displayed reduced water loss rate compared with wild-type plants (Figure 9c). In addition, the overexpression lines showed lower electrolyte leakage (Figure 9d). Taken together, our results indicated that LcFIN1 overexpression plants showed increased freezing tolerance.

Overexpression of *LcFIN1* promotes ROS scavenging

Malondialdehyde (MDA) accumulation has been considered to be an important parameter in regard to abiotic stress in plant cells because of its biological function in alleviating lipid peroxidation (Du *et al.*, 2012). The effects of low temperature on plant MDA contents were investigated in *LcFIN1*-transgenic and wild-type plants. MDA contents were significantly increased in both transgenic and wild-type plants when exposed to cold stress (Figure 9e). However, MDA accumulation levels were significantly reduced in *LcFIN1*-transgenic plants ($P < 0.01$). Lower MDA and electrolyte leakage levels in *LcFIN1*-transgenic plants implied reduced oxidative injury under cold stress, prompting us to assay the activities of three major antioxidant enzymes, SOD, CAT and POD. Under normal conditions, only one transgenic line showed significantly enhanced SOD compared with WT plants ($P < 0.05$). However, following 2 days of 4 °C treatment, SOD activities in all three transgenic plants were significantly higher than those of wild-type plants ($P < 0.05$) (Figure 9f). These results suggested that *LcFIN1*-transgenic plants inhibited ROS damage under cold stress by decreasing MDA accumulation and enhancing antioxidant enzyme activity.

FIN1 is positively regulated by CBF1

To understand why *FIN1* was rapidly induced by cold stress, we analysed the *LcFIN1* promoter, aiming to find the motif/*cis*-elements targeted by other known cold-stress-response transcription factors. Using the PLACE program (http://www.dna.affrc.go.jp/PLACE/signalscan.html), we found barley CBF1 binding sites (AT/GCCGAC) in the upstream promoter region (Figure 10a). As sheepgrass shares high homology with barley, the predicted CBF1 binding sites may also represent CBF1 binding sites in sheepgrass *LcFIN1* promoter region, suggesting that *LcFIN1* may be directly regulated by LcCBF1. To test this hypothesis, we first examined the *LcCBF1* expression pattern under cold stress. Quantitative RT-PCR showed than *LcCBF1* was also rapidly induced by cold stress, with peak level after 0.5-h stress treatment (Figure 10b). Given the even more rapid induction of *LcCBF1* by cold stress, it is likely that LcCBF1 is able to positively regulate *LcFIN1* expression.

To determine whether LcCBF1 protein can directly bind to the *LcFIN1* promoter, we used electrophoretic mobility shift assay (EMSA) to test the interaction between LcCBF1 and the *LcFIN1*

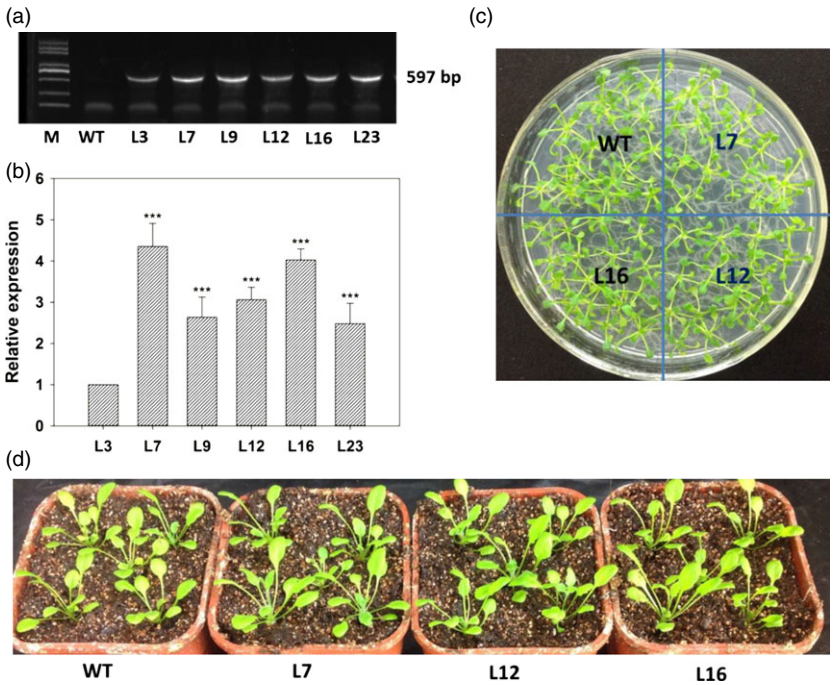


Figure 5 Phenotype of *LcFIN1* overexpression plants under normal conditions. (a) *LcFIN1* expression level revealed by RT-PCR in wild-type (WT) and several *LcFIN1-OE* plant samples (L3, L7, L9, L12, L16 and L23). The band (597 bp) represents specific *LcFIN1* in transgenic *Arabidopsis*. M, marker. (b) Expression of *LcFIN1* in transgenic *Arabidopsis* revealed by quantitative RT-PCR. Error bars indicate standard deviations of three technical replicates. (c) Phenotypes of T3 generation on 1/2 MS medium for 10 days. (d) Phenotypes of T3 generation wild-type (WT) and *LcFIN1* overexpression (*LcFIN1-OE*) plants grown in soil for 20 days. Asterisks represent statistically significant differences between L3 and other transgenic lines (L7, L9, L12, L23) by *t*-test (***$P < 0.001$).

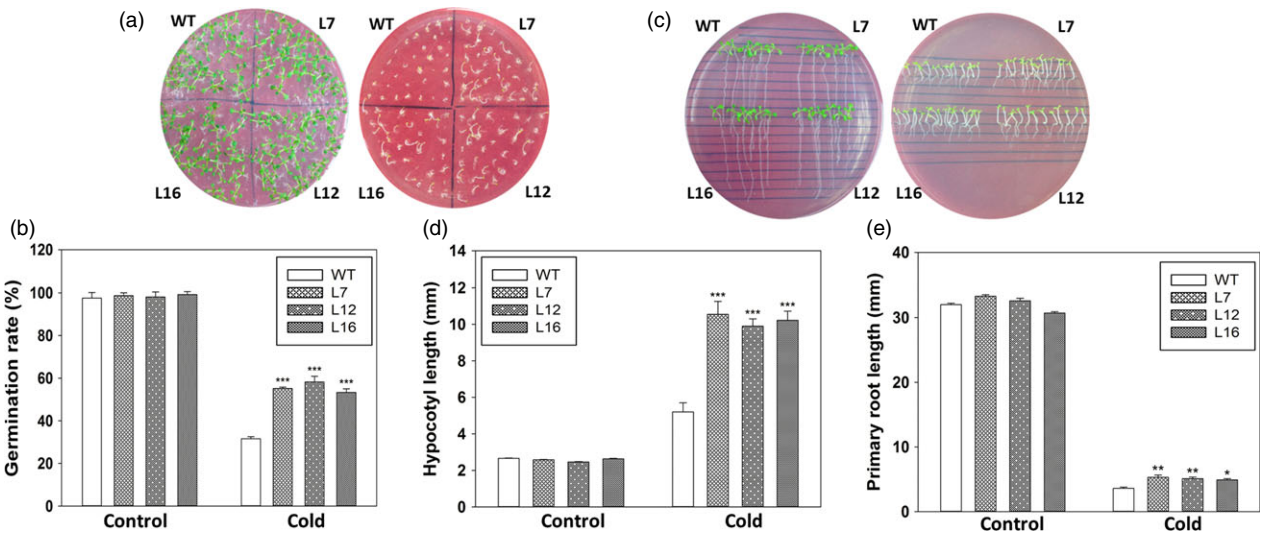


Figure 6 Chilling tolerance of *LcFIN1* overexpression (*LcFIN1-OE*) plants at germination stage. (a) Germination of wild-type and *LcFIN1-OE* plants. Left, plants grown at 22 °C for 7 days; right, plants placed at 4 °C for 35 days. (b) Quantification of germination rate for plants in (a). (c) Hypocotyl elongation of wild-type and *LcFIN1-OE* plants. Left, plants grown at 22 °C for 10 days; right, plants grown at 22 °C for germination and then transferred to 4 °C for 70 days. (d) Quantification of hypocotyl elongation of plants in (c). (e) Measurements of primary root length in WT and transgenic plants. Error bars indicate standard deviation of three independent biological replications. Asterisks indicate statistically significant differences between wild-type and *LcFIN1-OE* plants by *t*-test (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$).

promoter. As the result, a shifted DNA protein complex band was detected with the presence of CBF1-His protein, and no shift was detected in the absence of the CBF1-His protein (Figure 10c). The protein-bound band was also abolished with the addition of increasing amounts of unlabelled competitor DNA (Figure 10c). Taken together, these results indicate that LcCBF1 can directly bind to the *LcFIN1* promoter to regulate *LcFIN1* expression.

To confirm the interaction between CBF1 and *LcFIN1* promoter, chromatin immunoprecipitation (ChIP) assays were further conducted to test the protein–DNA interaction *in vivo*. The CHIP-qPCR results showed that *LcFIN1* promoter was specifically enriched by anti-LcCBF1 (Figure 10d) in sheepgrass after cold stress, suggesting that LcCBF1 can bind to *LcFIN1*'s promoter *in vivo*.

LcFIN1 positively regulates cold and oxidant stress-responsive genes

To assess the effects of *LcFIN1*-mediated transcriptional regulation in *Arabidopsis*, its transcriptome was analysed by RNA-sequencing technology, aiming to identify differentially expressed genes between transgenic and wild-type plants in cold condition (Figure 11a; Figure S1; Table S2). Among the 111 genes up-regulated in *LcFIN1* transgenic plants, we found two COR genes

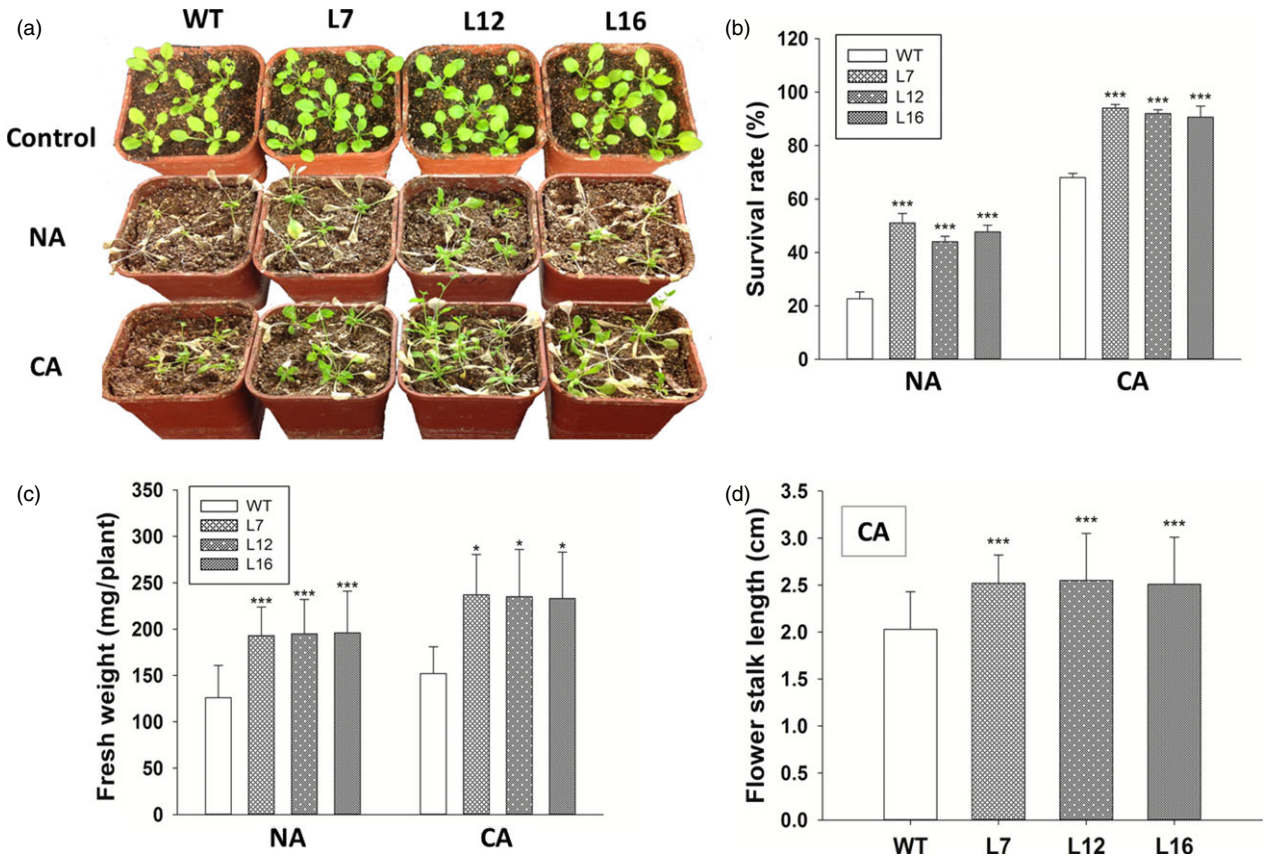


Figure 7 Freezing tolerance of *LcFIN1-OE* plants at vegetative stage. (a) Phenotypes of wild-type and *LcFIN1-OE* plants after freezing stress. Three-week-old plants were transferred to −8 °C (nonacclimated, NA) overnight, or following 4 °C treatment for 3 days (cold acclimated, CA). Phenotype was documented after 2 weeks of recovery at 22 °C. (b) Survival rate of *LcFIN1-OE* plants in (a) after freezing. Surviving plants capable of regrowth were scored after 2 weeks recovery. (c) Fresh weight of *LcFIN1-OE* plants in (a) after freezing. Surviving plants that were capable of regrowth were measured after 2 weeks of recovery. (d) Flower stalk elongation of cold-acclimated plants in (a) after freezing. Flower stalk height was measured for survived plants after 2 weeks of recovery. Error bars indicate standard deviation and results are from three independent biological replications. Asterisks indicate statistically significant differences between WT and *LcFIN1-OE* plants by *t*-test (*$P < 0.05$ and ***$P < 0.001$).

(*COR15A* and *KIN1*) and one G-box regulating factor (*RCI2A*), which are reported to be cold inducible (Artus *et al.*, 1996; Medina *et al.*, 2001; Miura *et al.*, 2007). Quantitative RT-PCR confirmed the up-regulation of these genes and other stress-related marker genes in the transgenic lines after cold stress (Figure 11b). Additionally, some ROS-responsive genes, for example ascorbate peroxidase, glutathione S-transferase and a homologue of antioxidant, showed elevated transcript levels in the plants overexpressing *LcFIN1*, suggesting that the enhanced cold tolerance in transgenic plants partially resulted from ROS pathways modulation.

Cold-induced genes in the transgenic plants also include basic chitinase, osmotin, glycine-rich protein, pathogenesis-related protein, trypsin family proteins containing PDZ domains and putative membrane lipoproteins, all of which have been implicated in both abiotic and biotic stress response. GO term enrichment analysis indicated that *LcFIN1*-induced genes are primarily involved in antioxidant activity, defence response and cold acclimation (Figure 11c). Interestingly, analysis of *cis*-elements indicated that nearly one-third of the up-regulated genes (36/111) contain the *DRE/CRT cis*-element in their promoter regions, suggesting that regulation of the downstream low-temperature-responsive genes by LcFIN1 is possibly mediated by the CBF pathway.

To test whether LcFIN1 can bind to the promoter of the downstream genes, ChIP-qPCR was performed to analyse the interaction between LcFIN1 and the promoter of the downstream genes. The results showed that the promoter of *AtKIN1* from Arabidopsis could be enriched by anti-LcFIN1 (Figure 11d), and there were no distinct differences in the enrichment of *AtCOR15A* and *AtRCI2A* promoters between normal conditions and cold, suggesting that LcFIN1 is able to directly regulate the expression of cold-related gene *AtKIN1* and indirectly regulated genes *AtCOR15A* and *AtRCI2A*.

Discussion

Sheepgrass is distributed widely in northern China and is cold, salt and dehydration tolerant (Wang *et al.*, 2008). Transcriptome sequencing for sheepgrass subjected to salt, cold or dehydration stress has identified many stress-responsive genes (Chen *et al.*, 2013). In this study, a cold-responsive gene, *LcFIN1,* was studied. Bioinformatics analysis indicated that the FIN1 contains no known domains except for a DUF761 domain (Figure 1a), which is also of unknown function. Although highly similar homologues of *LcFIN1* have been widely found in monocotyledons such as *A. tauschii* (87%) and *T. urartu* (81%), all these genes are annotated as unknown protein, suggesting that FIN1 is a novel cold-responsive gene.

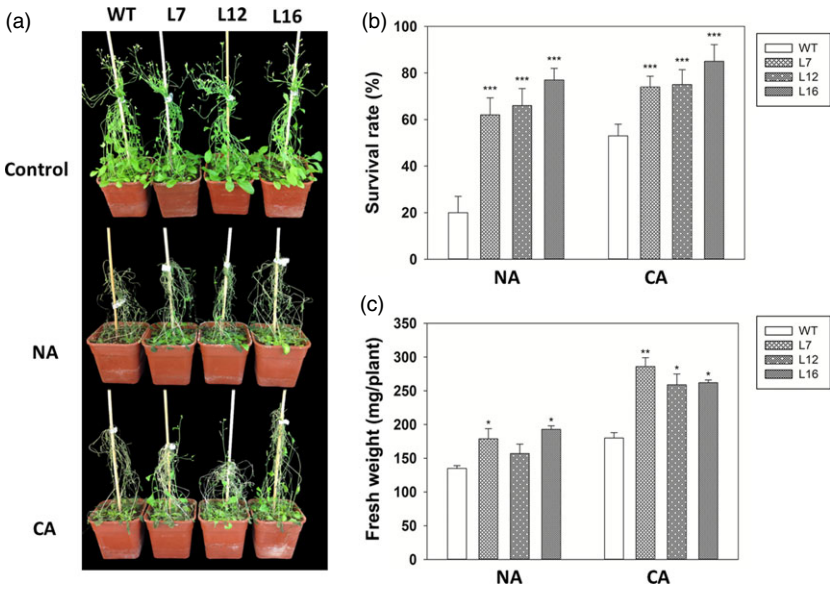


Figure 8 Freezing tolerance of *LcFIN–OE* plants at flowering stage. (a) Phenotypes of flowering wild-type and *LcFIN1-OE* plants after freezing stress. Six-week-old plants were transferred to −4 °C (nonacclimated, NA) overnight, or following 4 °C treatment for 24 h (cold acclimated, CA). Phenotype was documented after 10 days of recovery at 22 °C. (b) Fresh weights of transgenic and wild-type plants under normal growth condition. (c) Survival rate of *LcFIN1-OE* plants in (a) after freezing. Error bars indicate standard deviation of three independent biological replications. Asterisks indicate statistically significant difference between wild-type and *LcFIN1-OE* plants by *t*-test (*P < 0.05, **P < 0.01 and ***P < 0.001).

Expression analysis showed that transcripts of *LcFIN1* were significantly induced by cold stress (Figure 2c), whereas induction by salt, dehydration or ABA was not obvious (Figure 2b), suggesting that *LcFIN1* may play an important role specifically in response to cold stress rather than salt or dehydration stress. *LcFIN1* is different with previously reported *LcSAIN2*, *LcDREB3a* and *LcMYB1*, which were induced by ABA, salt and dehydration treatments (Cheng *et al.*, 2013; Li *et al.*, 2013a; Peng *et al.*, 2011).

Transcription factors (TFs), including CBFs, MYBs and ERFs, are localized in the nucleus and possess transcriptional activity to up/down-regulate downstream genes in response to biotic and abiotic stress (Cheng *et al.*, 2013; Oh *et al.*, 2007; Rong *et al.*, 2014; Seo *et al.*, 2011; Soltész *et al.*, 2013). LcFIN1-GFP fusion protein was clearly localized in the nucleus (Figure 3). Furthermore, LcFIN1 exhibited transcriptional activation activity in an *Arabidopsis* protoplast transient expression system (Figure 4). These results suggested that LcFIN1 may be a novel type of TF or cofactor of TFs, which acts as a positive regulator to promote plant cold tolerance.

To evaluate the role of *LcFIN1* in abiotic stress tolerance, we have attempted to generate transgenic sheepgrass as done before (Peng *et al.*, 2013; Wang *et al.*, 2009). Because genetic transformation in sheepgrass is still very difficult currently, we failed to generate transgenic sheepgrass in this work. Therefore, we used *Arabidopsis* to overexpress *LcFIN1* (Figure 5). Our results indicated that *LcFIN1*-overexpression *Arabidopsis* showed enhanced tolerance to cold stress, displaying a higher germination rate, longer hypocotyls and higher survival rate than wild-type plants when exposed to low temperatures (Figures 6–8). Besides, transgenic lines overexpressing *LcFIN1* did not affect the development and growth under normal conditions, making it an ideal candidate for improving abiotic tolerance in crops such as rice and cotton through genetic engineering, like other genes we isolated from sheepgrass previously (Cheng *et al.*, 2013; Li *et al.*, 2013a,b; Ma *et al.*, 2014; Peng *et al.*, 2011, 2013).

To understand the mechanism of *LcFIN1* overexpression in responding to cold stimulus, several physiological processes were analysed in transgenic and wild-type plants. The accumulation of soluble sugar (Mostajeran and Rahimi-Eichi, 2009; Wanner and Junttila, 1999) is a common phenomenon under abiotic stress. The accumulated compatible solutes that act as osmolytes to retain water in cells and reduce the rate of water loss in response to environmental stress thus increase stress tolerance and protect plants (Dubey and Singh, 1999; Valentovič *et al.*, 2006). In this study, *LcFIN1*-overexpressing plants accumulated more soluble sugars and had reduced water loss, which may account for the higher tolerance of *LcFIN1*-overexpressing plants to cold stress (Figure 9b,c). In addition, enhanced plant tolerance to environmental stresses is associated with increased cell membrane stability and photosynthetic capacity (Mao *et al.*, 2012). In the present study, we observed increased chlorophyll content and reduced ion leakage in *LcFIN1* transgenic plants (Figure 9a,d), suggesting that *LcFIN1* plants may have higher photosynthetic capability and less membrane damage. Plant cell ROS accumulation damages cellular structures and macromolecules, ultimately leading to cell death (Overmyer *et al.*, 2003). *LcFIN1*-overexpressing plants showed low (MDA) contents (Figure 9e), which is widely recognized as an indicator of reduced harm to plant cells suffering from abiotic stress (Du *et al.*, 2012). Moreover, SOD activity in *LcFIN1*-overexpressing plants was markedly higher than in wild-type plants (Figure 9f), suggesting that the transgenic plants suffered from less severe oxidative stress under cold stress.

To further elucidate the molecular mechanism of *LcFIN1* in response to cold stress, the transcriptome from both transgenic and wild-type plant was analysed by sequencing. Notably, transcripts encoding LTPs (lipid transfer proteins), which have been reported to be involved in abiotic stress responses (Guo *et al.*, 2013; Seo *et al.*, 2011), were significantly higher in transgenic plants than in wild-type plants. Additionally, the expression levels of dehydrin and antioxidant genes were higher in transgenic plants than in wild-type plants, in agreement with the physiological data. The different expression levels of numerous cold-responsive genes (e.g. *AtKIN1, AtCOR15a* and *AtRCI2A*) between transgenic plants and wild-type plants were detected by transcriptome analysis (Figure 11b), suggesting that the improved cold tolerance of *LcFIN1*-overexpressing plants may result from *LcFIN1*-mediated regulation on cold-responsive genes, such as *AtKIN1* and *COR15a*. *AtKIN1* is significantly up-regulated by low

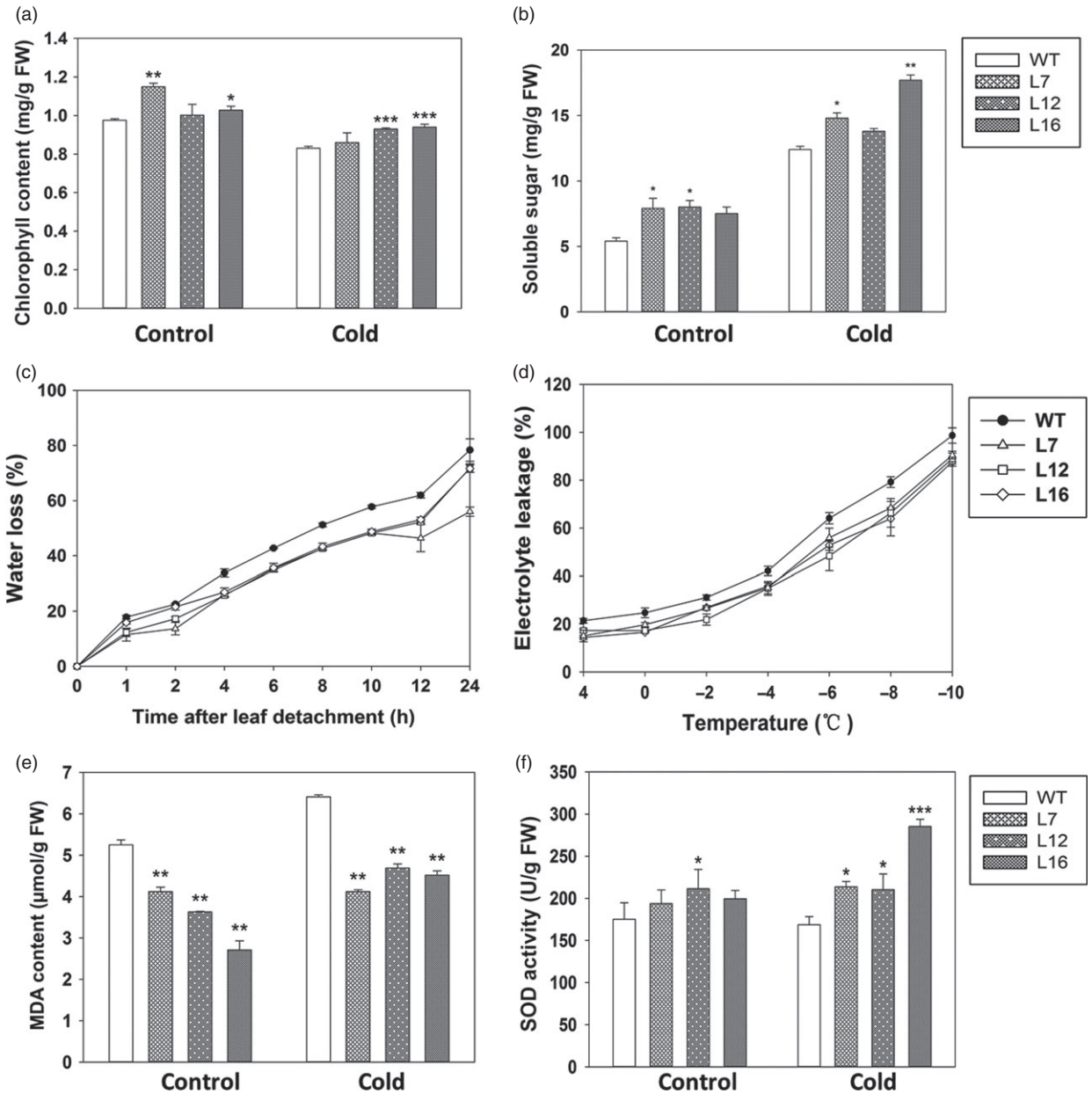


Figure 9 Metabolite measurements for wild-type and *LcFIN1-OE* plants. (a,b) Chlorophyll and soluble sugar contents of wild-type and *LcFIN1-OE* plants, respectively. Two-week-old plants grown at 22 °C (control) were exposed to 4 °C for 12 h (cold) and sampled for measurement of chlorophyll and soluble sugar contents. (c) Comparison of water loss rate for detached rosettes from wild-type and *LcFIN1-OE* plants. Leaves from 3-week-old plants grown at 22 °C were detached and weighed at designated time intervals. Fresh weight loss was calculated by the relative weight to the initial weight. (d) Electrolyte leakage of wild-type and *LcFIN1-OE* plants. (e,f) Oxidant content (e) and antioxidant enzymes levels (f) in wild-type and *LcFIN1-OE* plants after cold stress. Two-week-old plants grown in 22 °C (control) were exposed to 4 °C for 2 days (cold) before sample collection and determination of malondialdehyde (MDA) contents and superoxide dismutase (SOD) activity. Error bars indicate standard deviation of three independent replications. Asterisks indicate statistically significant difference between wild-type and *LcFIN1-OE* plants by *t*-test (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$).

temperature, osmotic and dehydration stress (Kasuga *et al.*, 1999), while *COR15a* is a cold-regulated gene of *A. thaliana* encoding a chloroplast-targeted polypeptide (Artus *et al.*, 1996). Both *AtKIN1* and *COR15a* participate in the CBF cold-responsive pathway (Shinwari *et al.*, 1998). *Cis*-element analysis showed that the upstream region of *LcFIN1* contained the *CRT/DRE* motif which is the binding site of DREB1/CBFs (Figure 10a), suggesting that *LcFIN1* may be regulated by DREB1/CBFs. Consistent with this notion, the cold-mediated induction of *LcFIN1* in sheepgrass seedlings was later than that of *LcCBF1* (Figure 10b), and EMSA and ChIP results showed that LcCBF1 was able to bind to the *CRT/DRE cis*-element in the promoter region of the *LcFIN1* gene (Figure 10c,d). Thus, in sheepgrass, LcFIN1 may be directly activated by LcCBF1 through *CRT/DRE* element (Guo *et al.*, 2013; Rong *et al.*, 2014). The ChIP-qPCR results indicated that LcFIN1 can enrich the promoters of *AtKIN1* in LcFIN1-OE plants under cold stress, suggesting that LcFIN1 is able to directly regulate the expression of *AtKIN1* under cold stress (Figure 11d).

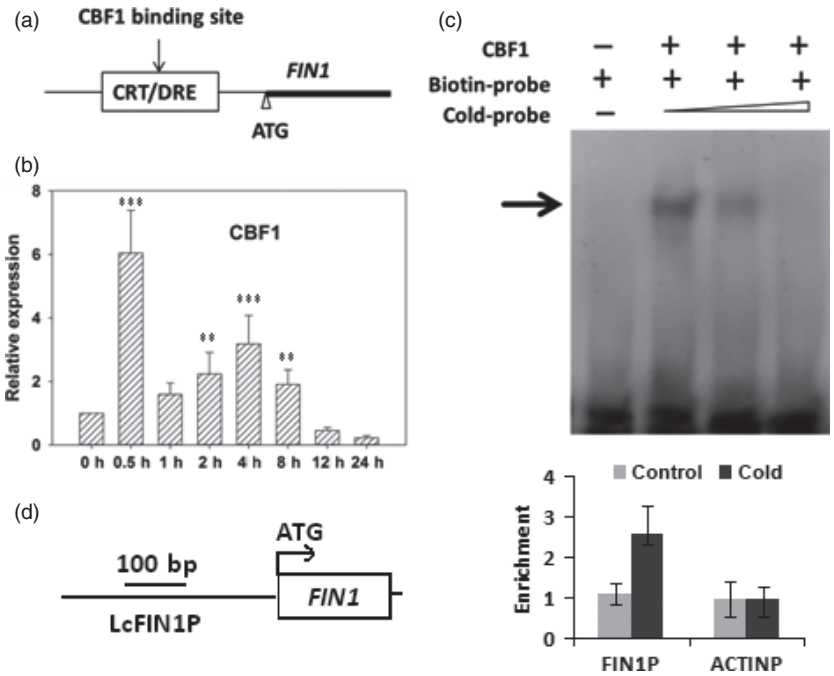
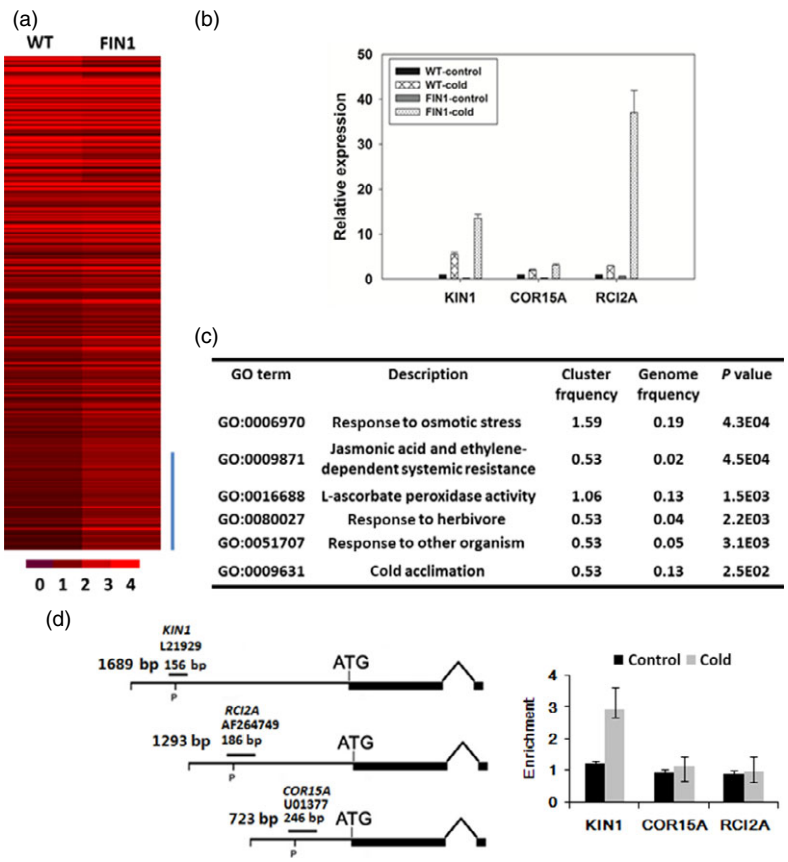


Figure 10 *FIN1* is directly regulated by *CBF1*. (a) Schematic diagrams showing *FIN1* promoter structure. The *CRT/DRE* motif contains a CBF1 binding site, and the translation start site (ATG) is indicated. (b) *CBF1* expression in response to cold treatment. Total RNA isolated from 14-day-old sheepgrass seedlings grown at 4 °C was used for time course expression analysis. Error bars indicate standard deviations of three technical replicates. (c) Electrophoretic mobility shift assay (EMSA) showing the interaction between CBF1 and *FIN1* promoters. Biotin-labelled DNA fragments were incubated with FIN1-His protein. A competition assay was performed by addition of excess of unlabelled DNA. The arrow indicates shifted band which was abolished by the presence of competitor. (d) ChIP-qPCR analysis of the enrichment of *LcFIN1* promoter by anti-CBF1 antibody. The accumulation of PCR product in control and cold of sheepgrass was normalized to *LcACTIN*. Asterisks indicate statistically significant difference between control and cold treatments by *t*-test (***P* < 0.01 and****P* < 0.001).

The increased expression of *LcFIN1* and LcFIN1-regulated stress-related genes led to physiological changes in cells, such as accumulation of soluble sugar and chlorophyll, increased cell membrane stability and antioxidant enzyme activity, which further contributed to cellular homeostasis and improved tolerance to cold stress as described above.

It is noteworthy that, although the overexpression of *AtCBF/DREB1* improved tolerance to drought, salt and freezing by activating the expression of stress-related genes, the overexpression plants showed a dwarf phenotype (Kasuga *et al.*, 1999; Liu *et al.*, 1998). The strong expression of the downstream stress-related genes is responsible for the dwarf phenotype of *AtCBF* OE plants under normal conditions (Kasuga *et al.*, 1999). In *LcFIN1*-OE plants, the expression of these cold-related genes (*KIN1*, *COR15A* and *RCI2A*) was lower in *LcFIN1*-OE plants compared with WT plants under normal conditions. We speculated that is the reason why *LcFIN1*-OE



GO term	Description	Cluster frquency	Genome frquency	*P* value
GO:0006970	Response to osmotic stress	1.59	0.19	4.3E04
GO:0009871	Jasmonic acid and ethylene-dependent systemic resistance	0.53	0.02	4.5E04
GO:0016688	L-ascorbate peroxidase activity	1.06	0.13	1.5E03
GO:0080027	Response to herbivore	0.53	0.04	2.2E03
GO:0051707	Response to other organism	0.53	0.05	3.1E03
GO:0009631	Cold acclimation	0.53	0.13	2.5E02

Figure 11 *LcFIN1* positively regulates a large proportion of stress-responsive genes. (a) Hierarchical clustering of up-regulated genes that were differentially expressed in wild-type and *LcFIN1-OE* plants. Genes with increased expression in cold conditions (blue bar) in *LcFIN1-OE* plants are indicated. (b) Quantitative RT-PCR analysis of cold-responsive genes. Total RNA isolated from wild-type and *LcFIN1-OE* plants grown at 4 °C for 3 days was used for analysis. Error bars indicate standard deviations of three technical replicates. (c) GO category enrichment for genes with increased expression in cold-treated *LcFIN1-OE* plants. *P*-values indicate significant differences between the percentages of all genes (genome frequency) assigned to the GO categories compared to the percentage of cold-inducted cluster genes (cluster frequency) assigned to GO categories. A cut-off value of $P < 0.05$ was used. (d) ChIP analysis of the enrichment of promoter of *KIN1*, *COR15A* and *RCI2A* by anti-FIN1 antibody. The accumulation of PCR product in control and cold of overexpression FIN1 plants after immunoprecipitation with anti-FIN1 antibody has been normalized to *ACTIN2*. The amplification sites used for each target gene were indicated as 'P' (promoter). The data are determined from at least three fully independent experiments.

plants show normal growth phenotype under normal condition. It has been reported that the level of *DREB1/CBF* mRNA was correlated with the phenotypic changes of growth retardation of the 35S: DREB1 transgenic plants (Liu *et al.*, 1998). The *RD29A*, *KIN1*, *COR15A* and *COR47* genes were strongly expressed under control conditions in 35S:*AtCBF/DREB1* OE plants, but weakly expressed in RD29A: *AtCBF/DREB1* OE plants (Kasuga *et al.*, 1999). Therefore, we concluded that the reason why 35S:*LcFIN1*-OE plants showed no growth retardation was the low expression of the stress-induced genes under unstressed conditions.

Although the expression of *KIN1*, *COR15A* and *RCI2A* was lower under normal conditions, they were dramatically up-regulated under cold treatment, contributing to the enhanced cold tolerance. Given the different regulation pattern under normal condition and cold treatment, we speculated that, similar to the previously reported case, a protein can function as both activator and repressor to regulate two normally consecutive developmental programmes (Hoecker *et al.*, 1995). FIN1 may play dual roles to regulate the downstream gene expressions with respect to different environmental conditions in transgenic Arabidopsis. Future works of the detailed molecular mechanism study will provide more insights to understand the function of FIN1.

In summary, this study identified a novel transcript factor *LcFIN1* from sheepgrass (tolerance to low temperature −47.5 °C or lower), which functions as a positive regulator in response to cold stress. The overexpression of *LcFIN1* in *Arabidopsis* led to higher germination rates, longer hypocotyls and elevated survival rates through the accumulation of compatible osmolytes, maintenance of cell membrane stability, minimization of oxidative damage and up-regulation of stress-responsive genes during cold stress. *LcFIN1* is different from our previous reports of stress-tolerance genes isolated from sheepgrass; other genes such as *LcDREB3A*, *LcDREB2A*, *LcSAIN1*, *LcSAIN2*, *LcMYB1* and *LcWRKY5* function mainly in salt and drought stress, whereas *LcFIN1* rapidly and specifically responds to cold treatment and can improve the tolerance to cold stress in transgenic plants. We believe that these genes activate different regulations upon overexpression. For examples, the *LcSAIN1* gene might play a positive modulation role in increasing the expression of transcription factors (*MYB2* and *DREB2A*) and functional genes (*P5CS* and *RAB18*) in transgenic plants under salt stress (Li *et al.*, 2013b); *LcWRKY5* may play an important role in drought-response networks through regulation of the *DREB2A* pathway (Ma *et al.*, 2014); and *LcDREB2* contributes to the high resistance to salt and drought stress by cooperating with *LcSAMDC2* (Peng *et al.*, 2013). In 35S:LcFIN1-OE plants, *LcFIN1* changed some cold-stress-related genes (*KIN1, AtCOR15A and AtRCI2A*) in transgenic plants under cold stress. Taken together, these results indicate that *LcFIN1* is a promising genetic resource for the improvement of crop freezing tolerance.

Acknowledgements

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Hierarchical clustering of down-regulated genes that were differentially expressed in wild type and transgenic lines.
Table S1 Primer sequences used in this study.
Figure S2 ChIP analysis of the relative enrichment of promoter from some stress-induced genes by anti-FIN1 antibody in transgenic plants.
Table S2 Genes that were differentially expressed in wild type and *LcFIN1* overexpressing plants exposed to low temperature.